80 Rec'd PCT/PTO 18 JUN 1999

FORM PTO-1390 (REV. 1-98) U.S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
41826	
U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	
09/331262	

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/BR97/00081	19 December 1997	18 December 1996

TITLE OF INVENTION METHOD AND COMPOSITION FOR THE DIAGNOSIS OF EQUINE INFECTIOUS ANEMIA VIRUS DISEASE BY USING THE RECOMBINANT CAPSID PROTEIN VIRUA (P26)

APPLICANT(S) FOR DO/EO/US Paulo Cesar PEREGRINO FERREIRA, Erna Geessien KROON, Jenner Karlisson PIMENTA DOS REIS, Isabella Bias FORTES FERRAZ and Romulo CERQUEIRA LEITE

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [ ] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information:

Search Report.
International Preliminary Examination Report.
Inventor information sheet.

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17. [X] The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $970.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $840.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $760.00		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $670.00		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $96.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 970	
Surcharge of $130.00 for furnishing the oath or declaration later than [ ] 20 [X] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	$	
Total claims	3 - 20 =	0	x $18.00	$ 0	
Independent claims	1 - 3 =	0	x $78.00	$ 0	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $260.00	$	
TOTAL OF ABOVE CALCULATIONS =				$ 1,100	
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28). +				$	
SUBTOTAL =				$ 1,100	
Processing fee of $130.00 for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$ 1,100	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$	
TOTAL FEES ENCLOSED =				$ 1,100	
				Amount to be refunded:	$
				charged:	$

a. [X] A check in the amount of $ 1,100 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required by 37 CFR 1.16 and 1.17, or credit any overpayment to Deposit Account No. 25-0120. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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June 18, 1999

Benoit Castel
SIGNATURE

Benoit Castel
NAME

35,041
REGISTRATION NUMBER

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80 Rec'd PCT/PTO 18 JUN 1999

PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Paulo Cesar PEREGRINO FERREIRA et al.

Serial No. (unknown)

Filed herewith

METHOD AND COMPOSITION FOR THE DIAGNOSIS OF EQUINE INFECTIOUS ANEMIA VIRUS DISEASE BY USING THE RECOMBINANT CAPSID PROTEIN VIRUS (P26)

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents

Washington, D.C. 20231

Sir:

Prior to the first Official Action and calculation of the filing fee, please substitute pages 1-7 of the specification as published, with pages 1-7 attached hereto and marked "AMENDED SHEET". Please also substitute Claims 1-3 as originally filed with Claims 1-3 as filed in the Article 34 amendment of February 22, 1999. These pages containing Claims 1-3 are marked "AMENDED SHEET" and are also attached hereto.

R E M A R K S

The above changes in the specification and claims merely place the national phase application in the same

condition as it was during Chapter II of the international phase.

Respectfully submitted,

YOUNG & THOMPSON

By 

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June 18, 1999

PCT/BR 97/0008

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METHOD AND COMPOSITION FOR THE DIAGNOSIS OF EQUINE INFECTIOUS ANEMIA VIRUS DISEASE BY USING THE RECOMBINANT CAPSID PROTEIN VIRUS (P26)

TECHNICAL FIELD OF THE INVENTION

The present invention relates to a method of detecting antibodies against core antigen of equine infectious anemia virus (EIAV), using as antigen the recombinant protein (p26) in immunoenzymatic assays. . More particularly, it relates to the use of recombinant protein p26 in kits for diagnosis of equine infectious anemia (EIA).

BACKGROUND TO THE INVENTION

The equine infectious anemia (EIA) is one of the oldest diseases caused by virus, having been described for the first time in France by LIGNEE, **Rec. Med. Vet.**, 20:30, 1843, and recognized as viral disease by VALLEE and CARRE. **Acad. Sci.** ,139:331-333,1904. The disease affects exclusively the members of the family **Equidae** presenting a worldwide distribution and of great economical importance consequently.

The EIA virus (EIAV) is classified as a lentivirus of the **Retroviridae** family (CHARMAN et al. **J. Virol.** 19(2):1073 -1076,1976), it is genetic and antigenically related to the other lentiviruses that are characterized by developing persistent infection in host. The EIA has played an especially important role in comparative virology and in the studies of the acquired immunodeficiency syndrome (AIDS). Besides their morphological identity, both viruses are similar in terms of nucleotide sequences that code for structural surfaces' proteins. This group of viruses present genetic and antigenic variants during persistent infections, which are associated to the immunresponse scape. (MONTAGNIER et al. **Ann. Virol.**, 135:119-134, 1984, MONTELARO et al. **J. Biol. Chem.**, 259:10539-10544,1984, RUSHLOW et al. **Virology**, 155:309-321, 1986, STREICHER et al. **J. Am. Med. Assoc.** 256:2390-2391, 1986, STOLER et al. **J. Am. Med. Assoc.** 256:2360-2364,1986 and HAHN et al. **Science**, 232:1548-1553, 1986.

The transmission of EIAV occurs mainly through bites of arthropod vectors (tabanideo) which inoculate the virus into the animal's blood stream

(mechanical transmission) when feeding themselves.The way of transmission is responsible for the high prevalence of EIA in areas favorable to the life cycle of vectors (ISSEL et al. **Vet.**. 17:251-286, 1988). The EIAV can also be transmitted by the placenta and colostro of mares with high virus levels, and by needles and surgical instruments contaminated with blood (COGGINS **Comparative diagnosis of viral diseases,NY**, 4:646-658, 1981) . The course of infection show different clinical forms of the disease (subacute, chronic and mainly inaparent or assimptomatic) in horses (ISSEL & COGGINS, **J. Am. Vet. Med. Assoc.** 174(7):727-33, 1979) , and the most prominent signs are the feverish episodes, hemolytic anemia, anorexia, fast weight loss and ventral edema.

The laboratory diagnosis plays a decisive role in the control and the prevalence of assymptomatic carriers, non conclusive and possibility to confuse clinical diagnosis with other trypanosomiases, pyroplasmosis, leptospirosis, hepatitis and parasites.

The diagnosis of EIAV has been done though the detection of specific antibodies against surface antigens of virus present in the serum of affected animals using the Coggins or agar gel diffusion test (U.S.Pat. nro.3,929,982 and U.S.Pat. No. 3,932,601). In the Coggins test the antigen and serum sample are placed side by side in an agarose gel plate. If EIA antibodies are present in the test serum, they will form a precipitin line when diffusing toward the agarose gel

This methodology is inherently insensitive since EIAV antigen preparation derived from spleen of infected animals or equine derme cultures cells may be contaminated with non-EIAV antigens during its preparation. Besides, antibodies against non-EIAV antigens may be present in the test serum and can react with the non-EIA antigens forming a variety of nonspecific precipitin lines. Even if, the prepared EIAV-antigen batches can be purified the Coggins test is laborious, time-consuming and demanding of considerable expertise in interpretation of results. The Coggins test procedure takes twenty-four to forty-eight hours for the formation of clearly visible precipitin lines delaying results.

Porter, U.S.Pat.No.4,806,467, discloses a method for detecting the EIA virus using a competitive enzyme-linked immunoabsorbent assay incorporating a purified viral antigen and a monoclonal antibody. To obtain the antigen, the

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EIA virus must first be cultured. The antigen used was the p26 capsid protein of the EIAV and was obtained through (purification of the cultured virus by a variety of means) well known in the art. The technique of virus tissue cultures increases the possibility of assay yeld false positive results since the virus may be contaminated with other forms of protein or oven another virus. Additionally, the EIAV is hard to culture, making the Porter's approach difficult for large scale production.

The use of a synthetic peptide in an enzyme linked immunosorbent assay for the detection of human immunodeficiency virus (HIV) was disclosed in Shoeman, R.L. et al, Analytical Biochemistry 161:370-379 (1987).

Darrel & Peisheng, tue U.S.Patent No. 5,427,907, discloses a method to use a synthetic peptide as the antigen in an immunoassay for the detection of antibodies against the equine inectious anemia virus in the serum of horses. This procedure includes only the search of some epitopes of a virus proteins.

It is an object of the present invention to provide an assay for the detection of the equine infectious anemia virus antibodies which may be fast, easily and quickly performed by using the stable recombinant envelope protein (rgp26) which may be produced in sufficient amounts at a low cost.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects, features and many attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1 shows schematically the method of diagnosis

Figure 2 shows the titration of positive and negative sera in Elisa with the recombinant protein p26 as antigen.

Figure 3 demonstrates the distribution of the optical density (OD) in Elisa with the recombinant protein p26 as antigen with 84 positive and 70 negative horse serum samples, previously tested by IDGA and ELISA using EIAV- antigen produced in cell cultures

DETAILED DESCRIPTION OF THE INVENTION

It is, therefore , an object of the present invention to provide a method of immunodiagnosis for EIA disease that uses the recombinant protein p26 derived from viral capsid of EIAV . The method consists of binding the recombinant antigen to solid supports (microtiter plates, tubes, beads or nitrocelullose or nylon papers or any kind that allow protein binding) and to proceed the analysis of the sera (presence of antibodies) from animals suspected of infection with the EIAV.

The recombinant protein p26 is added to a solid phase support and incubated for sufficient time to ensure that protein was bound to the support. The equine test sample is added the support and incubated for a period of time sufficient to permit any EIA antibodies are removed from sample.

Labeled conjugate is added which binds to the protein-antibody complex. Following enough time to allow such binding, any unbound labeled conjugate is removed by washing. labeled conjugate is added which binds to the protein-antibody complex. Following enough time to allow such binding, any unbound labeled conjugate is removed by washing. High level of bound conjugate indicates a positive result, which means presence of EIA viral antibodies. A low level of bound conjugate indicates a negative result which means absence or undetectable level of EIA viral antibodies.

A variety of commercially available solid phase supports may be used for protein binding. The direct binding of equine antibodies present in the test serum to the solid phase support is likely to result in a false positive reading. To prevent such binding, the blocking solution is used to fill any empty binding sites on the support which did not bind antibody protein. Any substance which will not react with EIA viral antibodies will function as a blocker. A conjugate is some species which will recognize and bind with the test serum EIA viral antibody.

The conjugate may be labeled using a variety of labeling means, including but not limited to: enzyme labeling, fluorescent labeling, and magnetic labeling. If enzymatic labeling is the labeling means chosen, the conjugate is labeled with an enzyme preferably select from the group consisting of horseradish peroxidase and alkaline phosphatase. Other enzymes may be used.

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When an enzyme label is used, the labeled conjugate is detected by adding an amount of a substrate which will recognize and react with the enzyme label to form a product that will produce a color change visible to the naked eye. The presence of color indicates a sufficient level of test serum antibodies to indicate infection. An absence of color is an indicator of a lack of infection, as the animal did not produce a significant number of antibodies to the virus. Hence, the labeled conjugate had few antibodies, if any, to bind with and was subsequently removed from the support. There are a variety of both peroxidase and phosphatase substrates which will react with horseradish peroxidase and alkaline phosphatase enzymes, respectively to form a colored product.

A preferred peroxidase substrate is an ortho-phenylenediamine/hydrogen peroxide solution. The intensity of the color of the product may be quantified using a spectrophotometer to read absorbance. However, measuring the absorbance is not necessary to obtain an accurate reading of the results of the assay.

The titration of positive and negative sera in Elisa with 1 μg recombinant protein p26 as antigen. (Figure 2) shows a detection of antibodies anti-p26 in the ELISA test using dilutions of the serum from 4 to 256 obtaining OD from 0,800 to 0.400 OD. The negative controls demonstrate that there are no non specific reaction.

The optical density obtained when sera from 84 positive and 70 negative horses were tested is presented on Fig. 3, showing the frequency of the different optical densities obtained

An animal was experimentally infected and its sera tested with the ELISA p26. Figure 4 shows that specific antibodies were detected seven days after the infection together with the appearance of fever.

In order that this invention may be better understood the follow examples for illustrative purposes only, are described. The examples illustrate the present invention and are not intended to limit it in spirit or scope.

EXAMPLE 1

The process can be understood better through the following description in consonance with the illustration Fig 1 where the binding of the antigen

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(recombinant protein p26) to the solid support (1), it is done by its dilution in carbonate buffer (Na_2CO_3 0,1-0,5 M; $NaHCO_3$ 0,1-0,5 M, pH 8,0-9,6), added in the concentrations of 0,01-1μg and incubated the temperature of 4-8°C for 18-24 hours in microtechnique plates, tubes or beads followed by eletrotransferred or transferred passively in the these concentrations to nitrocellulose or nylon supports. After binding of the antigen, the support was washed of 3 to 6 times with buffer solution (0.01-0.02 M NaH_2PO_4, 0.01-0.02 M Na_2HPO_4, 0.02-0.04M KCl, 0.85-0.9% NaCl pH 7.07-7.5) and then with 0,05-0,1% of tween 20 (Buffer-Tween). To block the inespecific sites of binding (2) the used support was incubated with block solution (skimmed powdered milk 1-5% bovine , 1-5% albumin or 1-5% casein in Tween buffer) for 30-60 min at 23°C-37°C. After a new wash of the support with Tween buffer, as described previously the positive and negative control and the serum samples were diluted in Tween buffer, to bound to the antigen linked to the solid support (3), and incubated at 23°C-37°C. After new wash of the support with Tween buffer, the conjugate was added, where the anti- equine imunoglobuline binds to the antibodies that are tied up to the antigens (4). Conjugate can be an equine anti-imunoglobuline conjugated to the enzyme peroxidase or any other enzyme as acetylcolinesterase, lactato desidrogenase, galactosidase, glicose oxidase, alkaline fosfatase, or another. This conjugate was diluted in Tween buffer in agreement with its title and added to the support with incubation for 23°C-37°C for 30-60 min. A new wash of the support with Tween buffer and the development of the reaction was proceeded (5) with the enzyme of the conjugate, transforms the substrate of colorless to a red-faced product. The developing solution is composed of the substrate of the enzyme used in the conjugate that for the peroxidase for example is the ortofenilenodiamino diluted in 0,1-0,2 M phosphate or citrate buffer, pH 5,0-8,0. After the color development, that is proportional to the concentration of specific antibodies in each sample, solution of acid was used (sulfuric acid) for stopping the reaction (6), where the acid interrupts the previous reaction. For the end result the measurement (7) of the color intensity formed in each reaction (sample) was made. This reading was made visually or in spectrophotometer, in absorbance, with a specific filter for the color formed by the developing solution.

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EXAMPLE 2

The kit for diagnosis of the EIAV may contain the following products:(a) the antigen recombinant p26 from EIA coated to the solid support (microplate, microtiter wells, tubes, capillary tubes, sticks, dispticks, beads) with different chemical composition (polystirene, polypropylene, polyethylene, polycarbonate, polyvinyl, polystyrene, latex, nitrocellulose, nylon; cellulose, polyacrylamyde, cross-linked dextran and microcrystalline glass (b) the anti-equine immunoglobulin conjugated with label that is selected from the group consisting of an enzyme, a fluorescent marker, avidin-biotin (c) the substrate for the label as orthophenilenodiamine and H_2O_2 (d) a blocking solution (0.01-0.02 M, NaH_2PO_4, 0,01-0.02M,Na_2HPO_4,0,02-0,04 M KCl, NaCl 0,85-0,9% pH 7,0-7,5), with 0,05-0,1% of Tween 20 and skimmed powdered milk 1-5% bovine ,albumin 1-5% or casein 1-5% (e) a diluent solution for specimen and conjugate (NaH_2PO_4 0,01-0,02 M, Na^2HPO_4 0,01-0,02 M, KCl 0,02-0,04 M, NaCl 0,85-0,9% pH 7,0-7,5), with 0,05-0,1% of Tween 20 and 1% skimmed powdered milk (f) a diluent solution for substrate 0,1 M Na_2HPO_4, 0,1 M $C_6H_8O_7$ pH 5,0 (f) stop solution 7N H_2SO_4 (g) wash solution (0,01-0,02 M NaH_2PO_4, 0,01-0,02 M Na_2HPO_4, 0,02-0,04 M KCl, 0,85-0,9% NaCl pH 7,0-7,5), with 0,05-0,1% of Tween 20 (h) positive control inactivated horse serum (I) negative control inactivated horse serum

While the present invention has been described in connection with an example, it will be understood that modifications and variations apparent to those ordinary skill in the art are within the scope of the present invention.

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WHAT IS CLAIMED IS:

1. An immunoenzymatic assay for detecting the presence of antibodies to the equine infectious anemia virus recombinant p26 capsid antigen in equine test samples comprising:

(a) the use of the recombinant p26 capsid protein from the equine infectious anemia virus

(b)binding the recombinant p26 capsid antigen to a solid support,

(c) reacting the bound antigen with a test sample of serum,

(d) removing the unbound test sample,

(e) reacting the bounded test antibody with a labeled antibody

(f) measuring the amount of bound antibody specific to the equine anemia infectious virus p26 capsid antigen in the test sample

2. The immunoassay according to claim 1, wherein said label is selected from the group consisting of an enzyme, a fluorescent marker, avidin-biotin.

3. The immunoassay according to claim 1, wherein said solid support is selected from the group consisting of polystyrene or polypropilene microtiter wells, polyethylene, polypropylene, polycarbonate, polyvinyl; polystyrene, or glass test tubes,capillary tubes,dipstcks, or beads; latex beads; nitrocellulose, nylon; cellulose, polyacrylamide, cross-linked dextran and microcrystalline glass.

METHOD AND COMPOSITION FOR THE DIAGNOSIS OF EQUINE INFECTIOUS ANEMIA VIRUS DISEASE BY USING THE RECOMBINANT CAPSID PROTEIN VIRUS (P26)

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The present invention relates to a method of detecting antibodies against core antigen of equine infectious anemia virus (EIAV), using as antigen the recombinant protein (p26) in immunoenzymatic assays. . More particularly, it relates to the use of recombinant protein p26 in kits for diagnosis of equine infectious anemia (EIA).

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EIA virus must first be cultured. The antigen used was the p26 capsid protein of the EIAV and was obtained through (purification of the cultured virus by a variety of means) well known in the art. The technique of virus tissue cultures increases the possibility of assay yeld false positive results since the virus may be contaminated with other forms of protein or oven another virus. Additionally, the EIAV is hard to culture, making the Porter's approach difficult for large scale production.

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The recombinant protein p26 is added to a solid phase support and incubated for sufficient time to ensure that protein was bound to the support. The equine test sample is added the support and incubated for a period of time sufficient to permit any EIA antibodies are removed from sample.

Labeled conjugate is added which binds to the protein-antibody complex. Following enough time to allow such binding, any unbound labeled conjugate is removed by washing. labeled conjugate is added which binds to the protein-antibody complex. Following enough time to allow such binding, any unbound labeled conjugate is removed by washing. High level of bound conjugate indicates a positive result, which means presence of EIA viral antibodies. A low level of bound conjugate indicates a negative result which means absence or undetectable level of EIA viral antibodies.

A variety of commercially available solid phase supports may be used for protein binding. The direct binding of equine antibodies present in the test serum to the solid phase support is likely to result in a false positive reading. To prevent such binding, the blocking solution is used to fill any empty binding sites on the support which did not bind antibody protein. Any substance which will not react with EIA viral antibodies will function as a blocker. A conjugate is some species which will recognize and bind with the test serum EIA viral antibody.

The conjugate may be labeled using a variety of labeling means, including but not limited to: enzyme labeling, fluorescent labeling, and magnetic labeling. If enzymatic labeling is the labeling means chosen, the conjugate is labeled with an enzyme preferably select from the group consisting of horseradish peroxidase and alkaline phosphatase. Other enzymes may be used.

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When an enzyme label is used, the labeled conjugate is detected by adding an amount of a substrate which will recognize and react with the enzyme label to form a product that will produce a color change visible to the naked eye. The presence of color indicates a sufficient level of test serum antibodies to indicate infection. An absence of color is an indicator of a lack of infection, as the animal did not produce a significant number of antibodies to the virus. Hence, the labeled conjugate had few antibodies, if any, to bind with and was subsequently removed from the support. There are a variety of both peroxidase and phosphatase substrates which will react with horseradish peroxidase and alkaline phosphatase enzymes, respectively to form a colored product.

A preferred peroxidase substrate is an ortho-phenylenediamine/hydrogen peroxide solution. The intensity of the color of the product may be quantified using a spectrophotometer to read absorbance. However, measuring the absorbance is not necessary to obtain an accurate reading of the results of the assay.

The titration of positive and negative sera in Elisa with 1 μg recombinant protein p26 as antigen. (Figure 2) shows a detection of antibodies anti-p26 in the ELISA test using dilutions of the serum from 4 to 256 obtaining OD from 0,800 to 0.400 OD. The negative controls demonstrate that there are no non specific reaction.

The optical density obtained when sera from 84 positive and 70 negative horses were tested is presented on Fig. 3, showing the frequency of the different optical densities obtained

An animal was experimentally infected and its sera tested with the ELISA p26. Figure 4 shows that specific antibodies were detected seven days after the infection together with the appearance of fever.

In order that this invention may be better understood the follow examples for illustrative purposes only, are described. The examples illustrate the present invention and are not intended to limit it in spirit or scope.

EXAMPLE 1

The process can be understood better through the following description in consonance with the illustration Fig 1 where the binding of the antigen

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(recombinant protein p26) to the solid support (1), it is done by its dilution in carbonate buffer (Na_2CO_3 0,1-0,5 M; $NaHCO_3$ 0,1-0,5 M, pH 8,0-9,6), added in the concentrations of 0,01-1μg and incubated the temperature of 4-8°C for 18-24 hours in microtechnique plates, tubes or beads followed by eletrotransferred or transferred passively in the these concentrations to nitrocellulose or nylon supports. After binding of the antigen, the support was washed of 3 to 6 times with buffer solution (0.01-0.02 M NaH_2PO_4, 0.01-0.02 M Na_2HPO_4, 0.02-0.04M KCl, 0.85-0.9% NaCl pH 7.07-7.5) and then with 0,05-0,1% of tween 20 (Buffer-Tween). To block the inespecific sites of binding (2) the used support was incubated with block solution (skimmed powdered milk 1-5% bovine , 1-5% albumin or 1-5% caseín in Tween buffer) for 30-60 min at 23°C-37°C. After a new wash of the support with Tween buffer, as described previously the positive and negative control and the serum samples were diluted in Tween buffer, to bound to the antigen linked to the solid support (3), and incubated at 23°C-37°C. After new wash of the support with Tween buffer, the conjugate was added, where the anti- equine imunoglobuline binds to the antibodies that are tied up to the antigens (4). Conjugate can be an equine anti-imunoglobuline conjugated to the enzyme peroxidase or any other enzyme as acetylcolinesterase, lactato desidrogenase, galactosidase, glicose oxidase, alkaline fosfatase, or another. This conjugate was diluted in Tween buffer in agreement with its title and added to the support with incubation for 23°C-37°C for 30-60 min. A new wash of the support with Tween buffer and the development of the reaction was proceeded (5) with the enzyme of the conjugate, transforms the substrate of colorless to a red-faced product. The developing solution is composed of the substrate of the enzyme used in the conjugate that for the peroxidase for example is the ortofenilenodiamino diluted in 0,1-0,2 M phosphate or citrate buffer, pH 5,0-8,0. After the color development, that is proportional to the concentration of specific antibodies in each sample, solution of acid was used (sulfuric acid) for stopping the reaction (6), where the acid interrupts the previous reaction. For the end result the measurement (7) of the color intensity formed in each reaction (sample) was made. This reading was made visually or in spectrophotometer, in absorbance, with a specific filter for the color formed by the developing solution.

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EXAMPLE 2

The kit for diagnosis of the EIAV may contain the following products:(a) the antigen recombinant p26 from EIA coated to the solid support (microplate, microtiter wells, tubes, capillary tubes, sticks, dispticks, beads) with different chemical composition (polystirene, polypropylene, polyethylene, polycarbonate, polyvinyl, polystyrene, latex, nitrocellulose, nylon; cellulose, polyacrylamyde, cross-linked dextran and microcrystalline glass (b) the anti-equine immunoglobulin conjugated with label that is selected from the group consisting of an enzyme, a fluorescent marker, avidin-biotin (c) the substrate for the label as orthophenilenodiamine and H_2O_2 (d) a blocking solution (0.01-0.02 M, NaH_2PO_4, 0,01-0.02M,Na_2HPO_4,0,02-0,04 M KCl, NaCl 0,85-0,9% pH 7,0-7,5), with 0,05-0,1% of Tween 20 and skimmed powdered milk 1-5% bovine ,albumin 1-5% or caseín 1-5% (e) a diluent solution for specimen and conjugate (NaH_2PO_4 0,01-0,02 M, Na^2HPO_4 0,01-0,02 M, KCl 0,02-0,04 M, NaCl 0,85-0,9% pH 7,0-7,5), with 0,05-0,1% of Tween 20 and 1% skimmed powdered milk (f) a diluent solution for substrate 0,1 M Na_2HPO_4, 0,1 M $C_6H_8O_7$ pH 5,0 (f) stop solution 7N H_2SO_4 (g) wash solution (0,01-0,02 M NaH_2PO_4, 0,01-0,02 M Na_2HPO_4, 0,02-0,04 M KCl, 0,85-0,9% NaCl pH 7,0-7,5), with 0,05-0,1% of Tween 20 (h) positive control inactivated horse serum (I) negative control inactivated horse serum

While the present invention has been described in connection with an example, it will be understood that modifications and variations apparent to those ordinary skill in the art are within the scope of the present invention.

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WHAT IS CLAIMED IS:

1. An immunoenzymatic assay for detecting the presence of antibodies to the equine infectious anemia virus recombinant p26 capsid antigen in equine test samples comprising:

(a) the use of the recombinant p26 capsid protein from the equine infectious anemia virus

(b)binding the recombinant p26 capsid antigen to a solid support,

(c) reacting the bound antigen with a test sample of serum,

(d) removing the unbound test sample,

(e) reacting the bounded test antibody with a labeled antibody

(f) measuring the amount of bound antibody specific to the equine anemia infectious virus p26 capsid antigen in the test sample

2. The immunoassay according to claim 1, wherein said label is selected from the group consisting of an enzyme, a fluorescent marker, avidin-biotin.

3. The immunoassay according to claim 1, wherein said solid support is selected from the group consisting of polystyrene or polypropilene microtiter wells, polyethylene, polypropylene, polycarbonate, polyvinyl; polystyrene, or glass test tubes,capillary tubes,dipstcks, or beads; latex beads; nitrocellulose, nylon; cellulose, polyacrylamide, cross-linked dextran and microcrystalline glass.

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Figure 1

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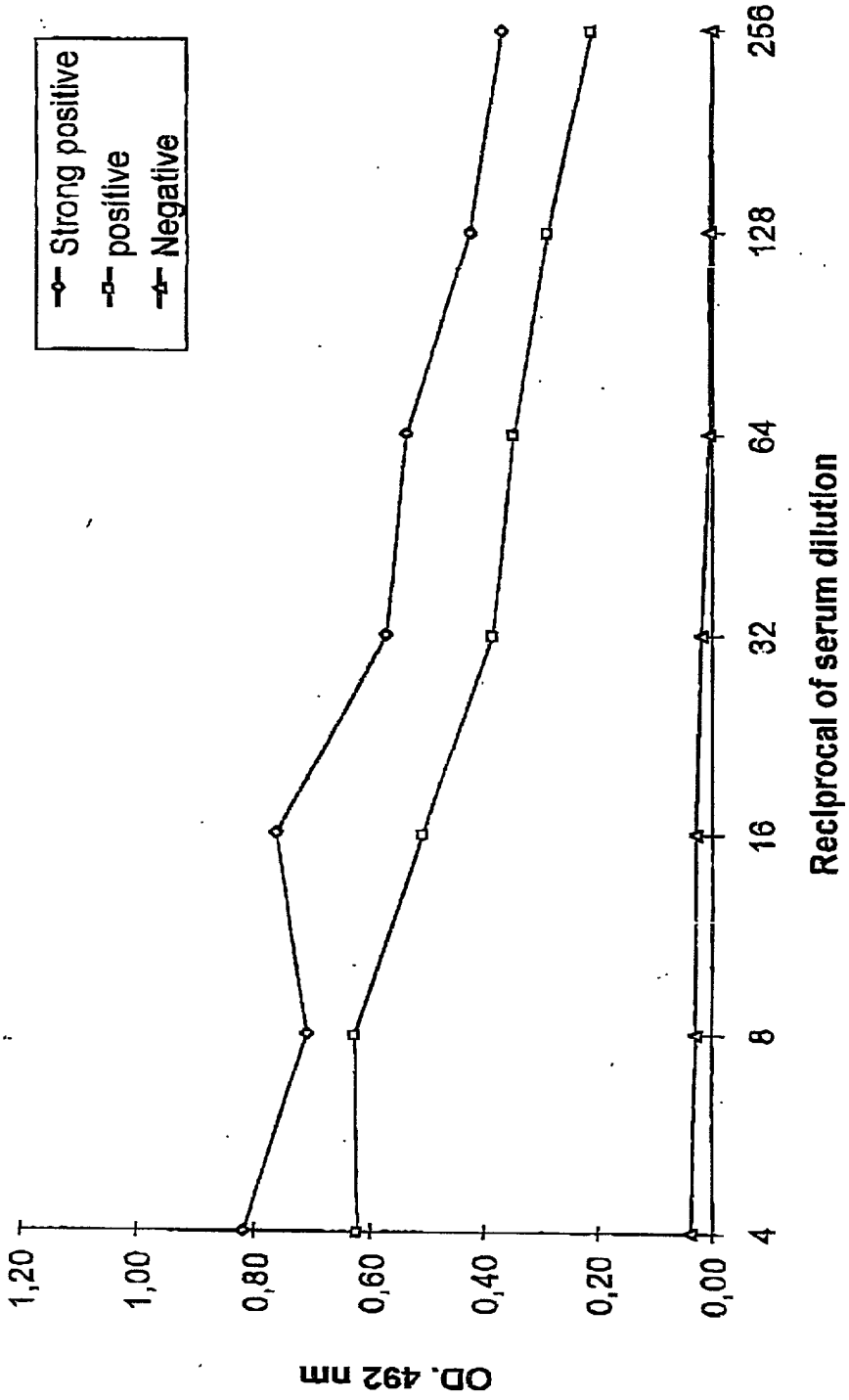

Figure 2

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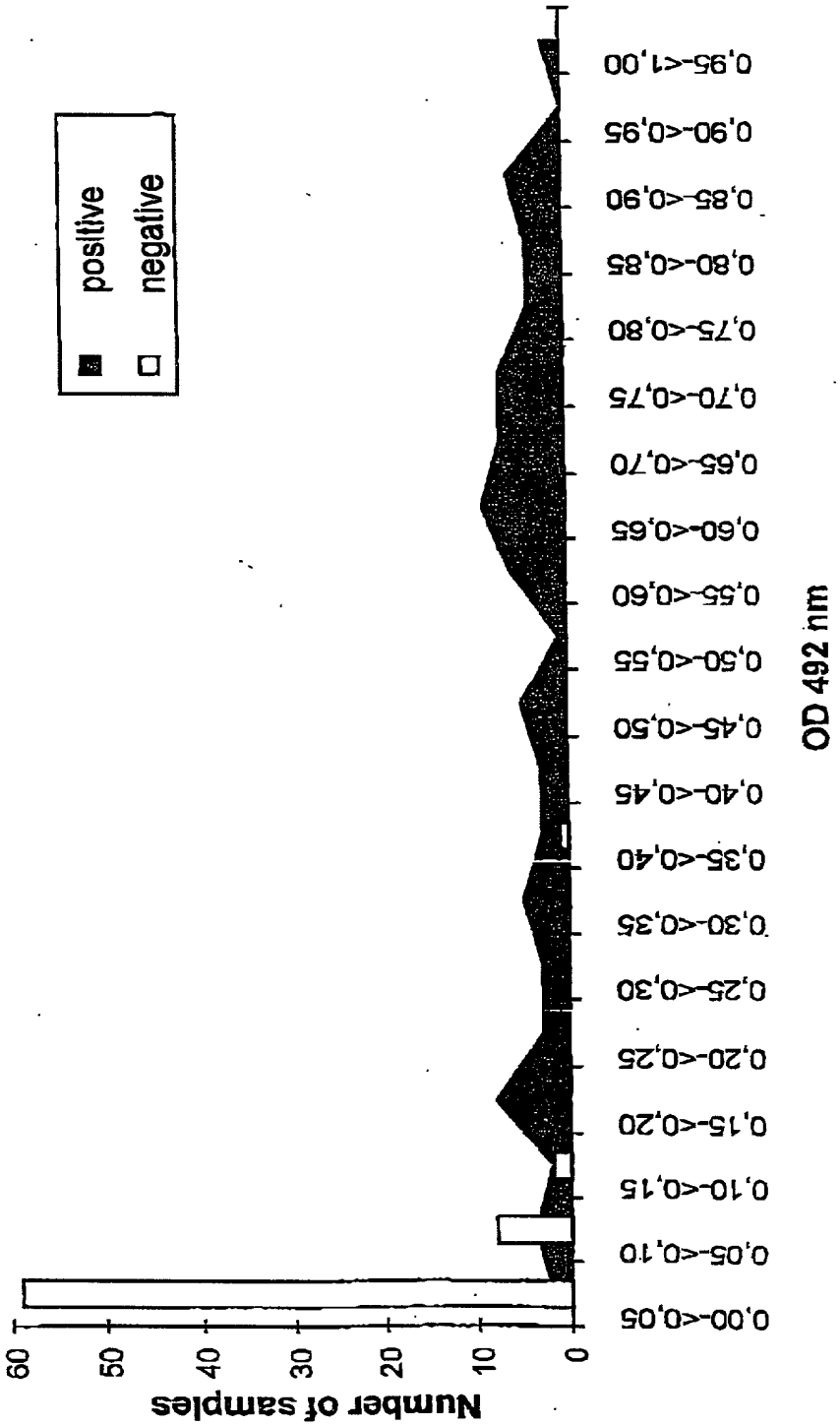
substitute sheet

Figure 3



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COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHOD AND COMPOSITION FOR THE DIAGNOSIS OF EQUINE INFECTIOUS ANEMIA VIRUS DISEASE BY USING THE RECOMBINANT CAPSID PROTEIN VIRUS (P26)

the specification of which: *(check one)*

REGULAR OR DESIGN APPLICATION

[ ] is attached hereto.

[ ] was filed on as application Serial No. and was amended on (if applicable).

PCT FILED APPLICATION ENTERING NATIONAL STAGE

[X] was described and claimed in International application No. PCT/BR97/00081 filed on 19 December 1997 and as amended on (if any).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

PRIORITY CLAIM

I hereby claim foreign priority benefits under 35 USC 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

PRIOR FOREIGN APPLICATION(S)

Country	Application Number	Date of Filing (day, month, year)	Priority Claimed
Brazil	18 December 1996	PI 9606273-8	yes

(Complete this part only if this is a continuing application.)

I hereby claim the benefit under 35 USC 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 USC 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.) (Filing Date) (Status--patented, pending, abandoned)

POWER OF ATTORNEY

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from Bergenstrahle & Lindvall AB as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

As a named inventor, I hereby appoint the following attorney(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: **Robert J. PATCH, Reg. No. 17,355, Andrew J. PATCH, Reg. No. 32,925, Robert F. HARGEST, Reg. No. 25,590, Benoît CASTEL, Reg. No. 35,041, Eric JENSEN, Reg. No. 37,855, Thomas W. PERKINS, Reg. No. 33,027, and Roland E. LONG, Jr., Reg. No. 41,949,** c/o YOUNG & THOMPSON, Second Floor, 745 South 23rd Street, Arlington, Virginia 22202.

Address all telephone calls to Young & Thompson at 703/521-2297.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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4-00

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